SUPPLEMENTARY INFORMATION

FOXO3 signalling links ATM to the p53 apoptotic pathway following DNA damage

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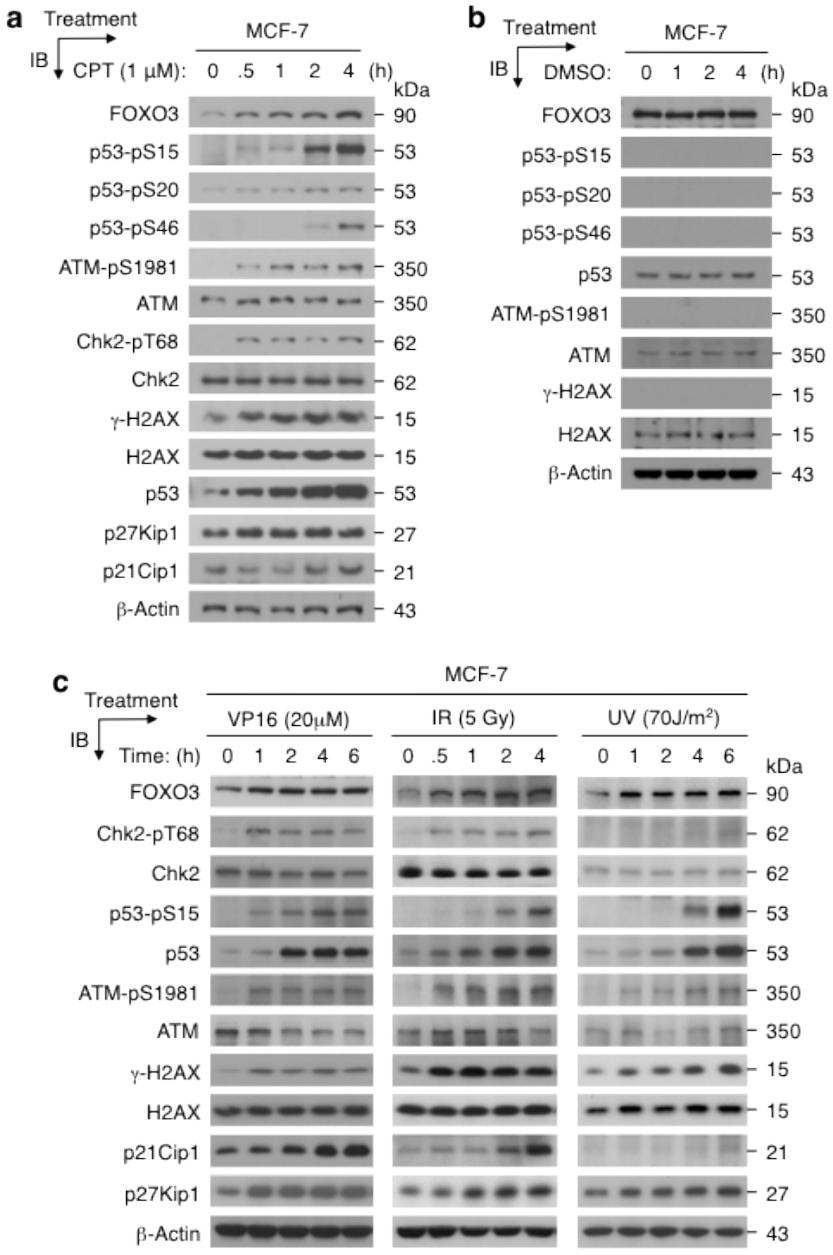
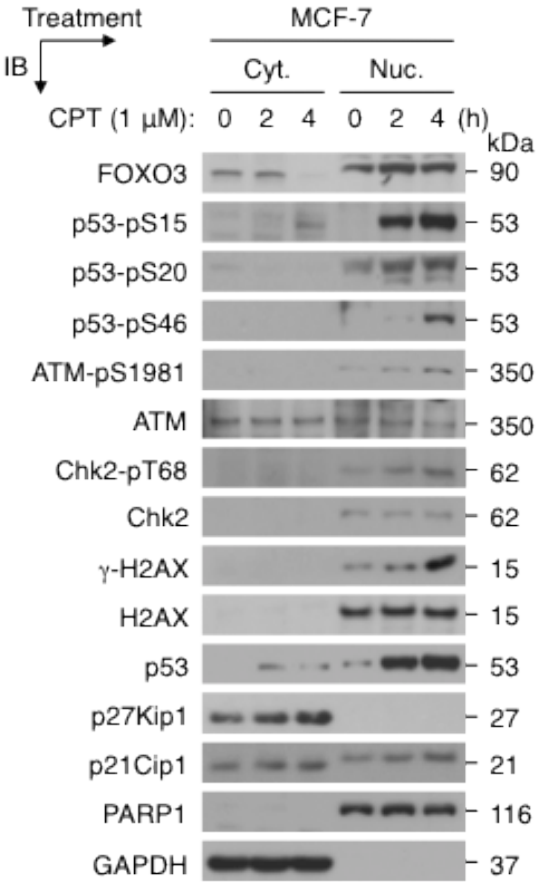
Supplementary Figures S1-S11

Supplementary Table S1

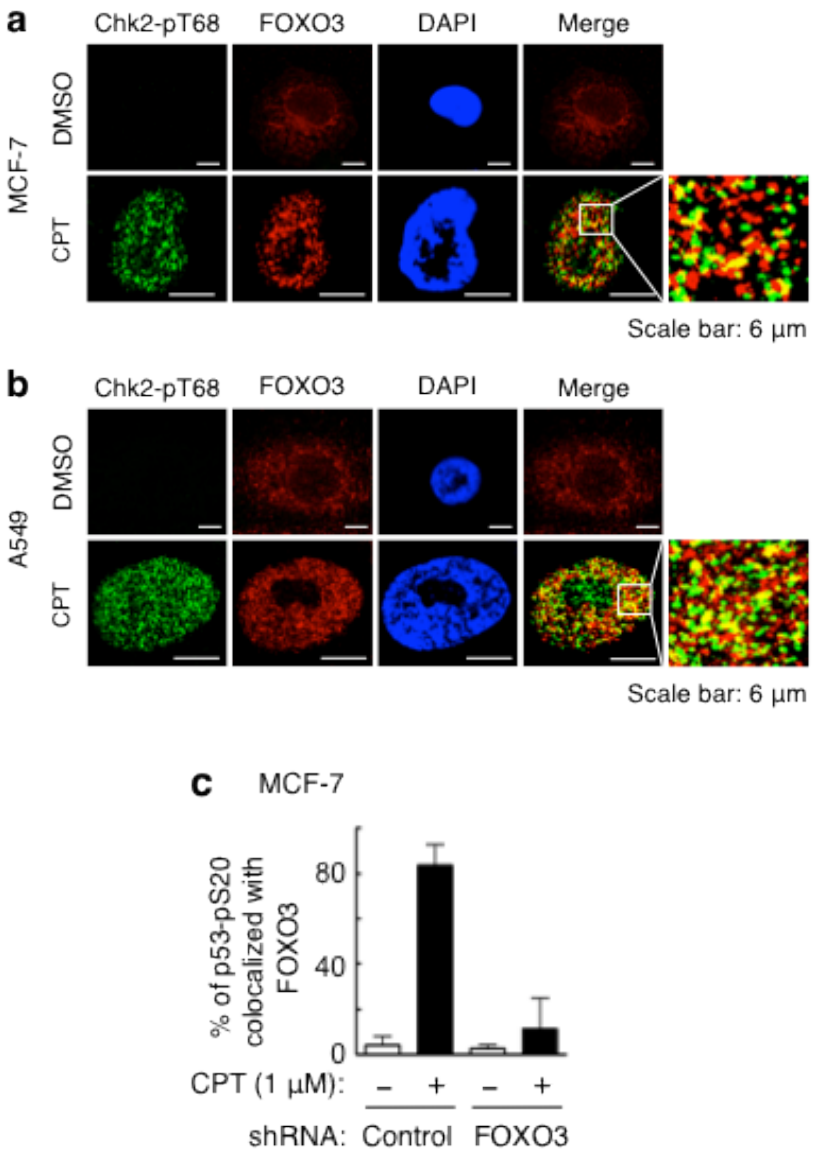
Supplementary Methods



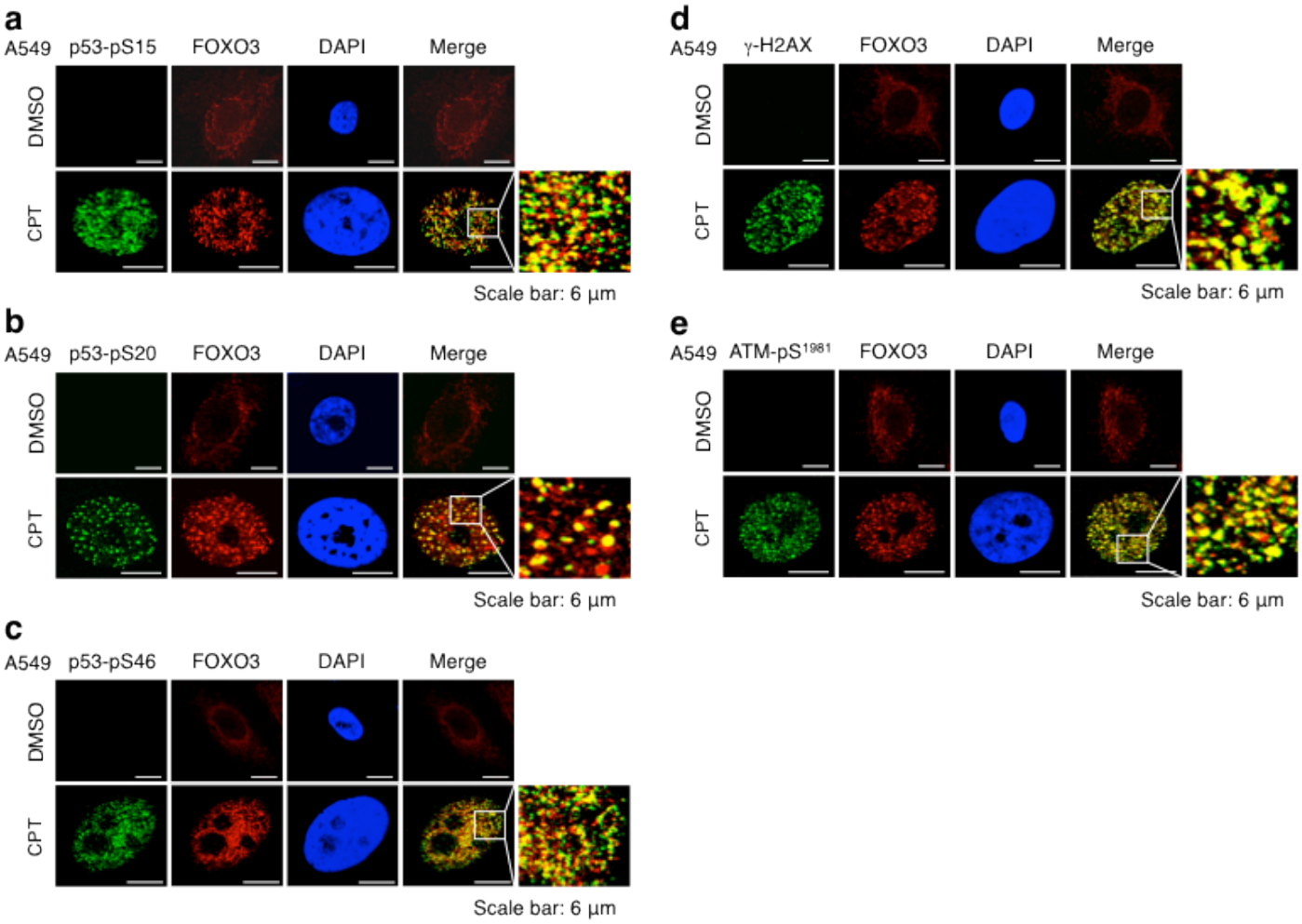
Supplementary Figure S1. (**a**) MCF-7 cells were treated with camptothecin (CPT) (1 μM) for various times, hours (h), as indicated. The levels of FOXO3, p53-pS15, p53-pS20, p53-pS46, Chk2-pT68, ATM-pS1981, γ-H2AX and their total proteins in whole lysates were analyzed by immunoblotting (IB) with specific phospho-antibodies (Abs) or Abs against whole proteins as highlighted. (**b**) As a negative control, IB analyses were carried out with whole lysates from MCF-7 cells treated with DMSO (control) for various times using Abs as indicated. (**c**) MCF-7 cells were treated with various DNA damaging agents, including epotoside (VP16, 20 μM), ionizing radiation (IR) (5 Gy), and UV (70J/m2) for various times as indicated. The levels of FOXO3, Chk2-pT68, p53-pS15, ATM-pS1981, γ-H2AX and their total proteins in whole cell lysates were analyzed by IB analysis using the indicated Abs as described above. p27Kip1 and p21Cip1 are the transcriptional targets of FOXO3 and p53, respectively. β-actin represents the loading controls of lysates.



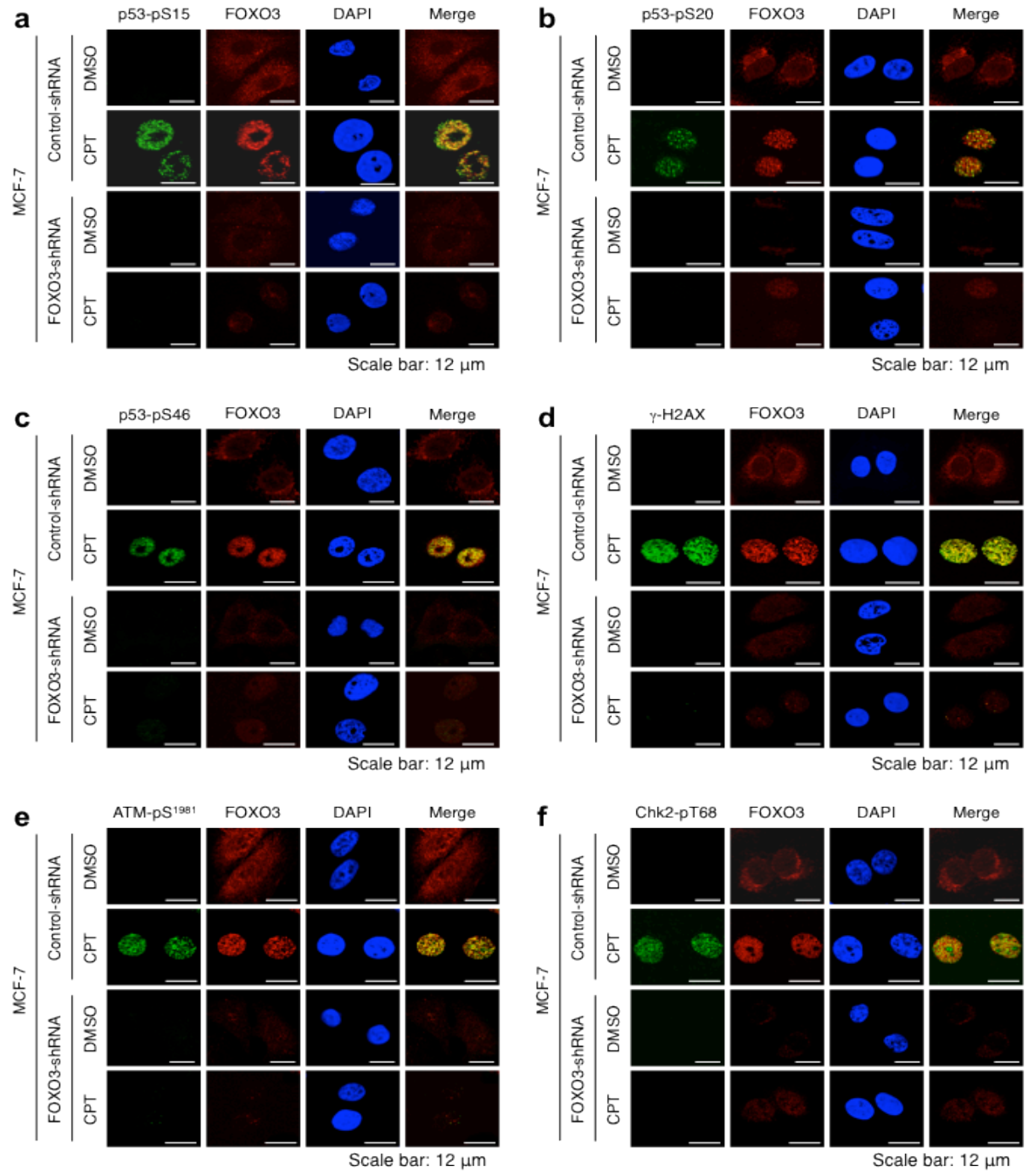
Supplementary Figure S2. DNA damage induced by CPT can increase nuclear FOXO3 and activate p53-pS15, p53-pS20, p53-pS46, Chk2-pT68, ATM-pS1981, and γ-H2AX in the nucleus concurrently. MCF-7 cells were treated with CPT (1 μM) for various times, and the levels of these test proteins and their downstream proteins in the cytoplamic (Cyt.) and nuclear (Nuc.) extracts were analyzed by immunoblotting (IB) with specific antibodies as indicated. As an expression control, the level of total expression of each protein was examined by IB analysis with an antibody against whole protein as indicated. The levels of p27Kip1 and p21Cip1, which are downstream targets of FOXO3 and p53, showed the activities of FOXO3 and p53, respectively. GAPDH and PARP1 represent the fractionation and loading controls of Cyt. and Nuc. extracts.



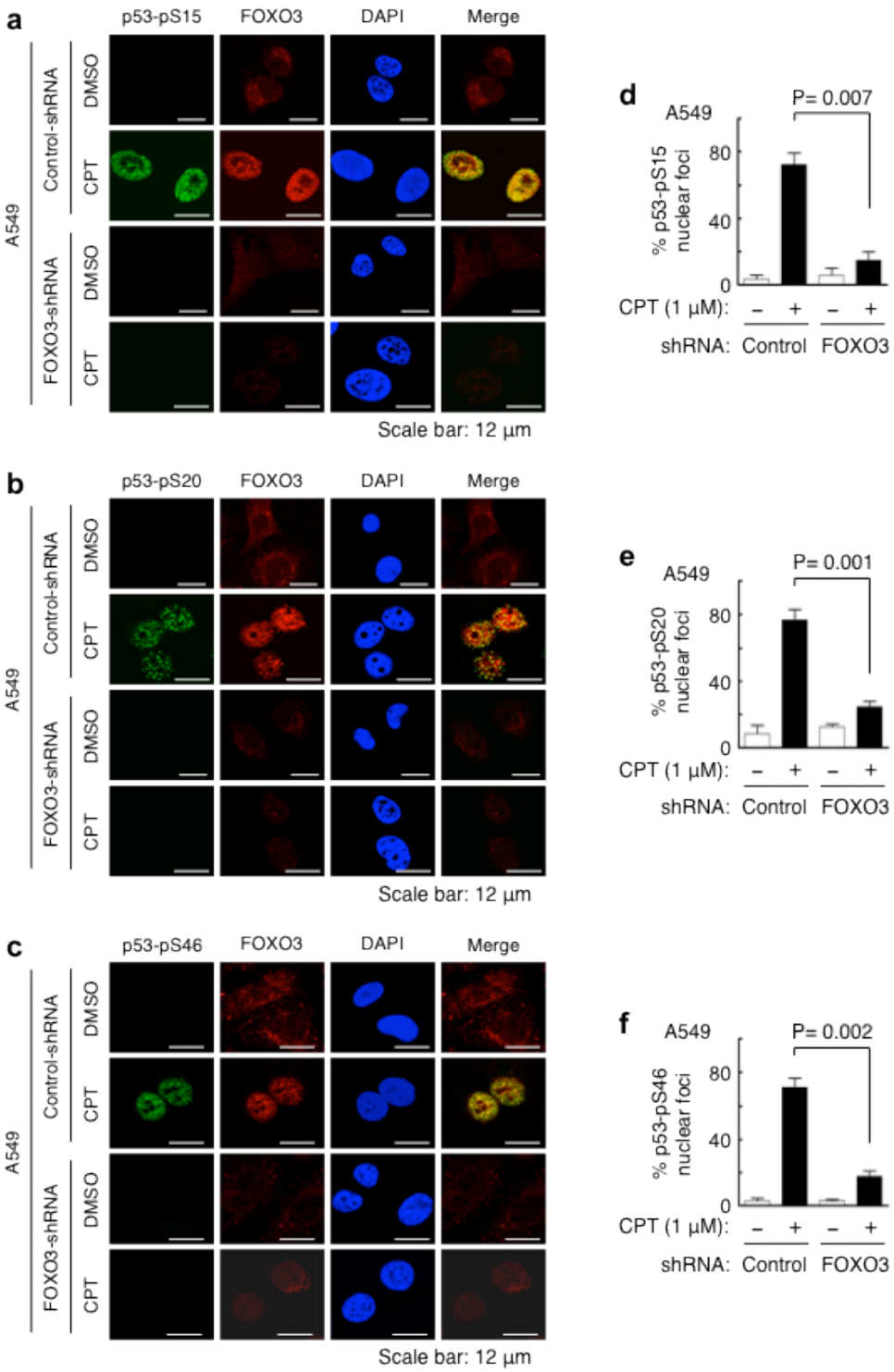
Supplementary Figure S3. Chk2-pT68 is colocalized with FOXO3 to form nuclear foci upon DNA damage induced by CPT. (**a**, **b**) MCF-7 (a) or A549 (b) cells were treated with CPT (1 μM) or DMSO control for 2 hours, fixed, and co-localizations between FOXO3 and Chk2-pT68 were stained with antibodies (Abs) against FOXO3 and Chk2-pT68, followed by an Alexa 594(red)- or Alexa 488(green)-conjugated secondary Ab, respectively, and fluorescence microscopy. DAPI was used to show the nuclei, and co-localizations of Chk2-pT68 with FOXO3 were shown as the merged images. Scale bar: 6 μm. (**c**) The percentage (%) of p53-pS20 colocalized with FOXO3 in MCF-7 cells (as shown in Fig 2c) was quantitated using the Velocity software (ver. 6.1, Improvision, PerkinElmer). The number of biological replicates is two, the error bars represent the range.



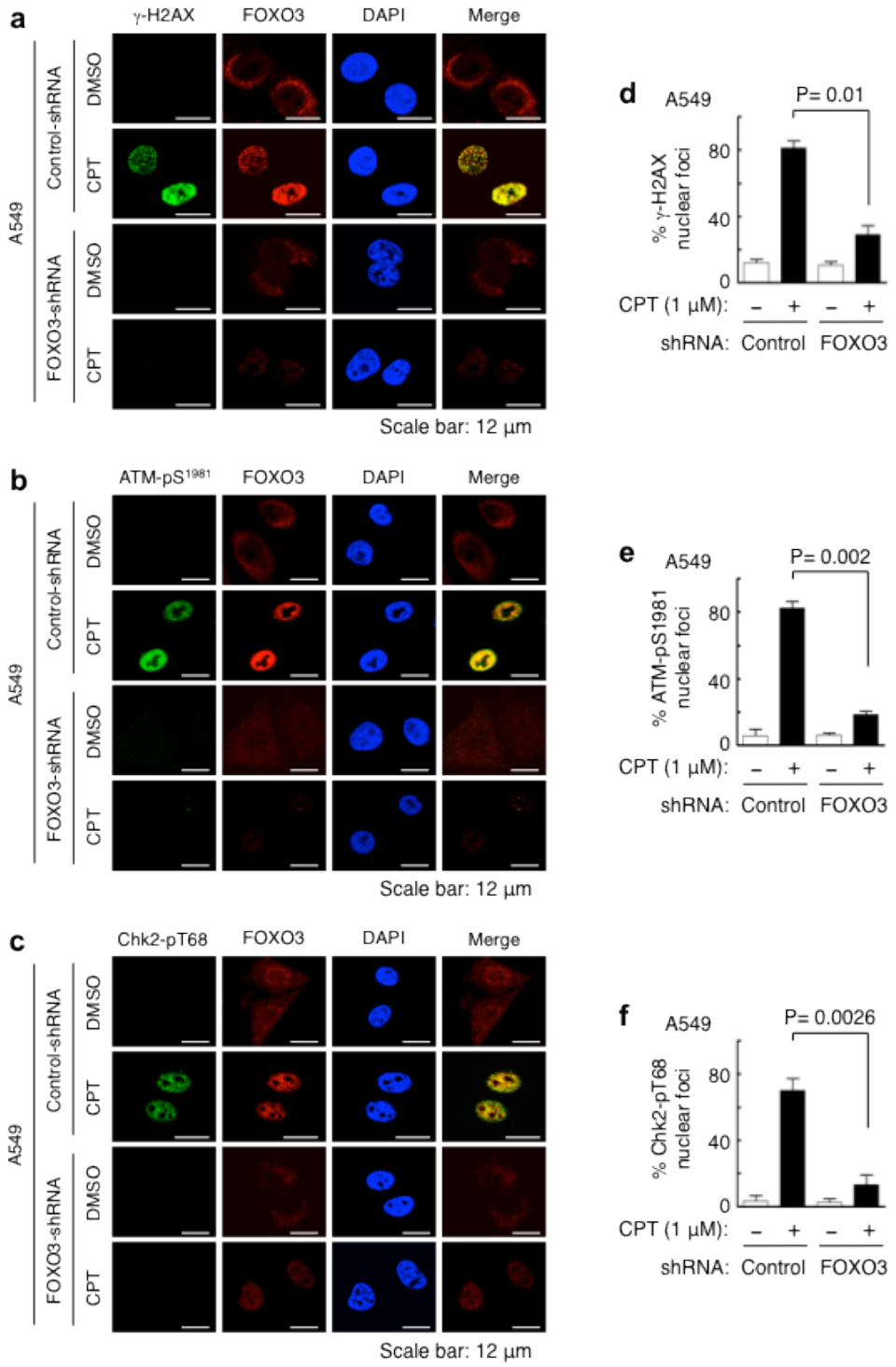
Supplementary Figure S4. FOXO3 is colocalized with ATM-pS1981, γ-H2AX, p53-pS15, p53-pS20, and p53-pS46 to form nuclear foci in A549 cells upon DNA damage induced by CPT. (**a**-**e**) A549 cells were treated with CPT (1 μM) or DMSO control for 2 h, fixed, and then the subcellular localizations and co-localization of endogenous FOXO3 and p53-pS15 (a) or p53-pS20 (b) or p53-pS46 (c) or γ-H2AX (d) or ATM-pS1981 (e) were detected using specific antibodies as indicated and followed by an Alexa Fluor 594(red)- or Alexa Fluor 488(green)-conjugated secondary antibody, and fluorescence microscopy. DAPI was used to show the nuclei. Localization of nuclear p53-pS15 or p53-pS20 or p53-pS46 or γ-H2AX or ATM-pS1981 was shown in green, FOXO3 was displayed in red, and co-localization of nuclear FOXO3 with each of them was exhibited as the merged yellow images. Scale bar: 6 µm.



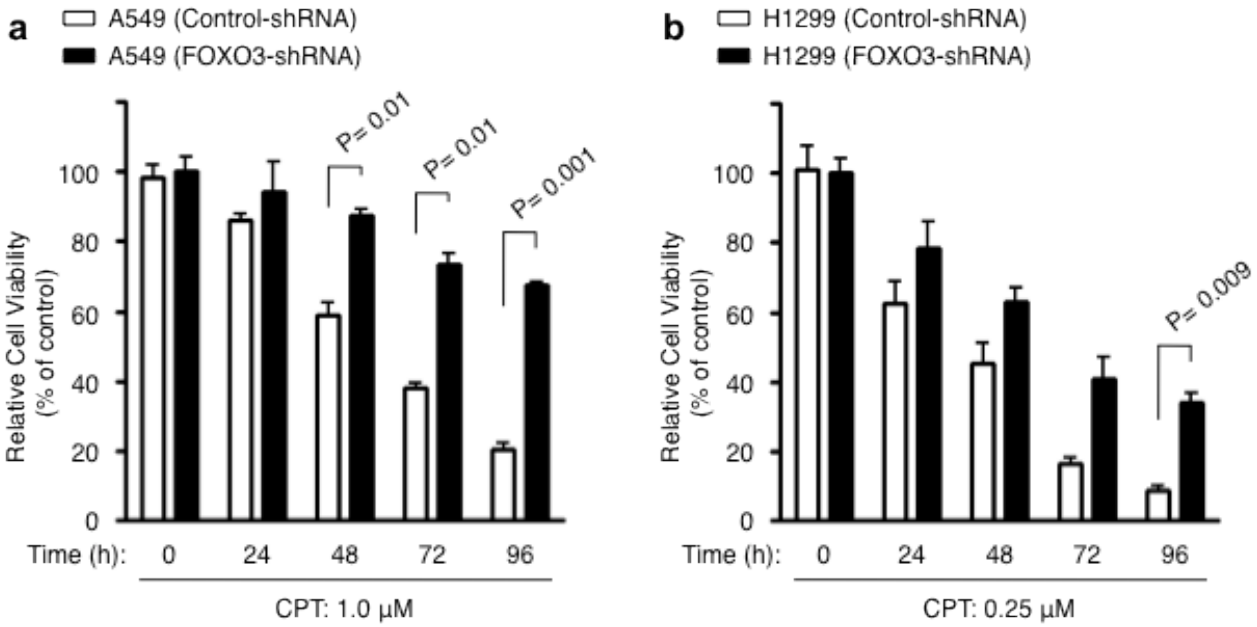
Supplementary Figure S5. (**a**-**f**) MCF-7 stable cell lines transfected with FOXO3-shRNA or control-shRNA were treated with CPT (1 μM) or DMSO for 2 h, and the subcellular localizations and colocalization of endogenous FOXO3 and p53-pS15 (a) or p53-pS20 (b) or p53-pS46 (c) or γ-H2AX (d) or ATM-pS1981 (e) or Chk2-pT68 (f) were detected using specific Abs as indicated and fluorescence microscopy. DAPI was used to show the nuclei. Co-localization of nuclear FOXO3 with p53-pS15 or p53-pS20 or p53-pS46 or γ-H2AX or ATM-pS1981 or Chk2-pT68 was exhibited as the merged yellow images. An average of 200 cells with specific nuclear foci in each comparison was determined and shown in Figure 2g-l. Scale bar: 12 μm.



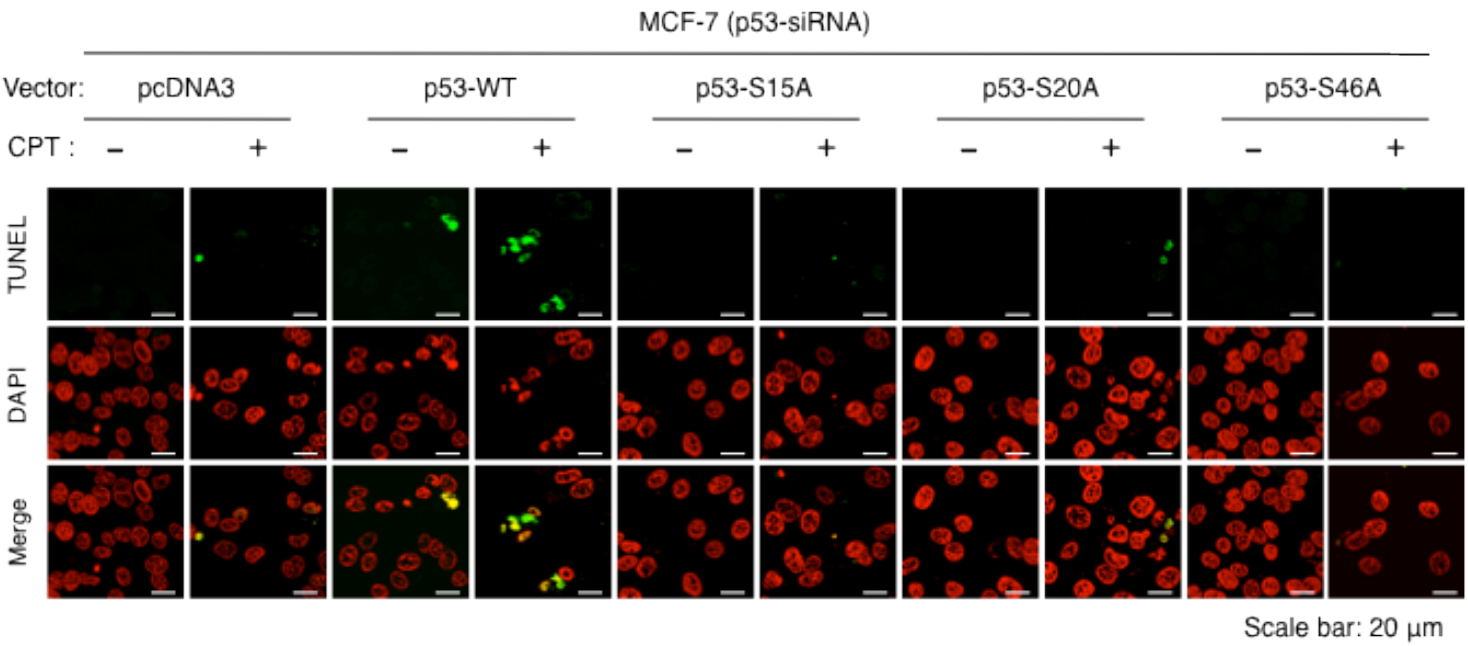
Supplementary Figure S6. (**a**-**c**) A549 (control-shRNA) and A549 (FOXO3-shRNA) stable cell lines were treated with CPT (1 μM) or DMSO for 2 h, and then the subcellular localizations and colocalization of FOXO3 and p53-pS15 (a) or p53-pS20 (b) or p53-pS46 (c) were detected using specific Abs as indicated and fluorescence microscopy. DAPI was used to show the nuclei. Scale bar: 12 μm. (**d**-**f**) An average of 200 cells with specific nuclear foci of p53-pS15 (d) or p53-pS20 (e) or p53-pS46 (f) in each comparison was determined and shown. The samples include three biological replicates, the error bars represent standard deviation, and the statistical test is the paired t-test.



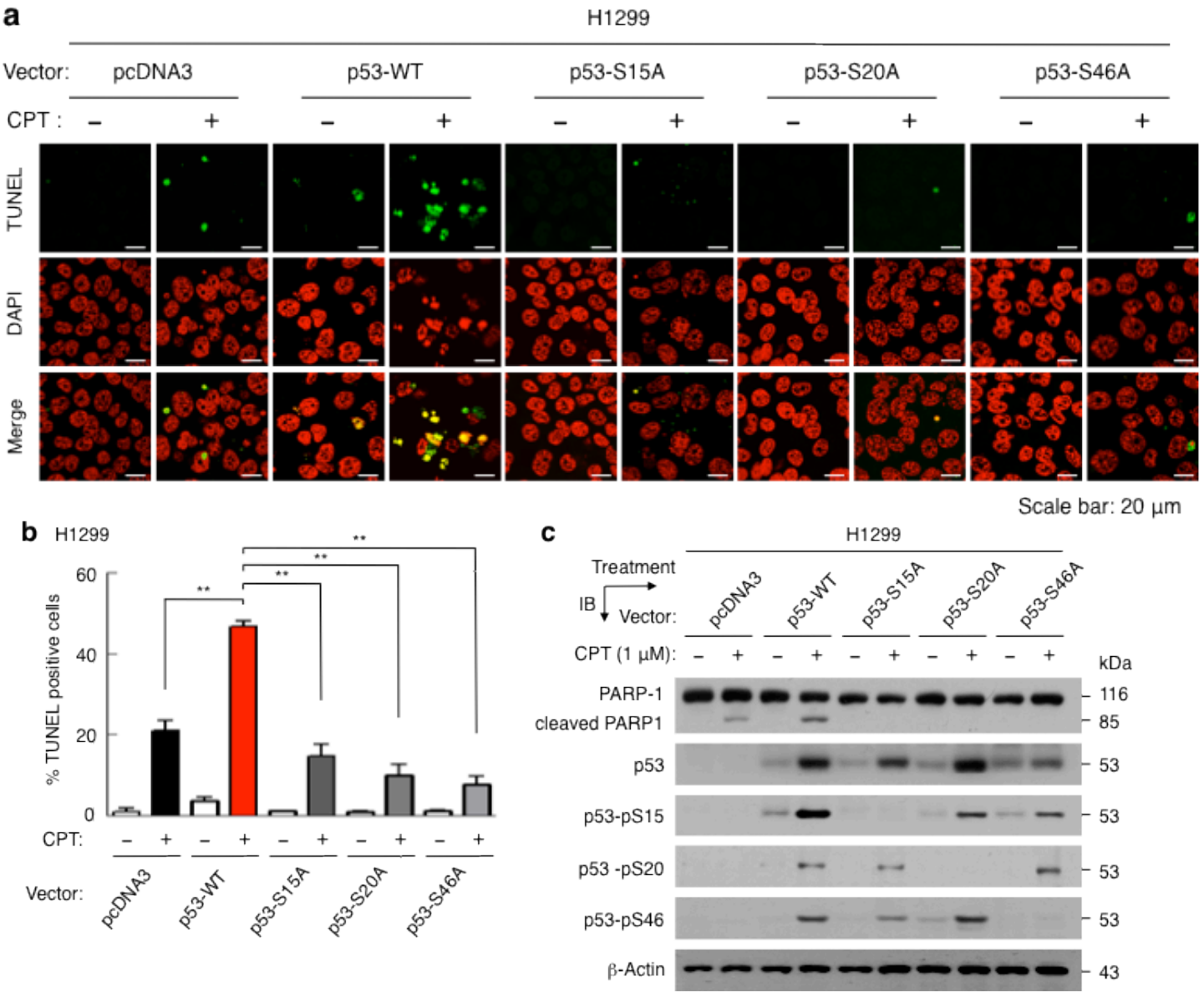
Supplementary Figure S7. (**a**-**c**) A549 (control-shRNA) and A549 (FOXO3-shRNA) stable cell lines were treated with CPT (1 μM) or DMSO for 2 h, and then the subcellular localizations and co-localization of FOXO3 and or γ-H2AX (a) or ATM-pS1981 (b) or Chk2-pT68 (c) were detected using the indicated Abs and fluorescence microscopy. DAPI was used to show the nuclei. Scale bar: 12 μm. (**d**-**f**) An average of 200 cells with specific nuclear foci of γ-H2AX (d) or ATM-pS1981 (e) or Chk2-pT68 (f) in each comparison was determined and shown. The number of biological replicates is three, the error bars represent standard deviation, and the statistical test is the paired t-test.



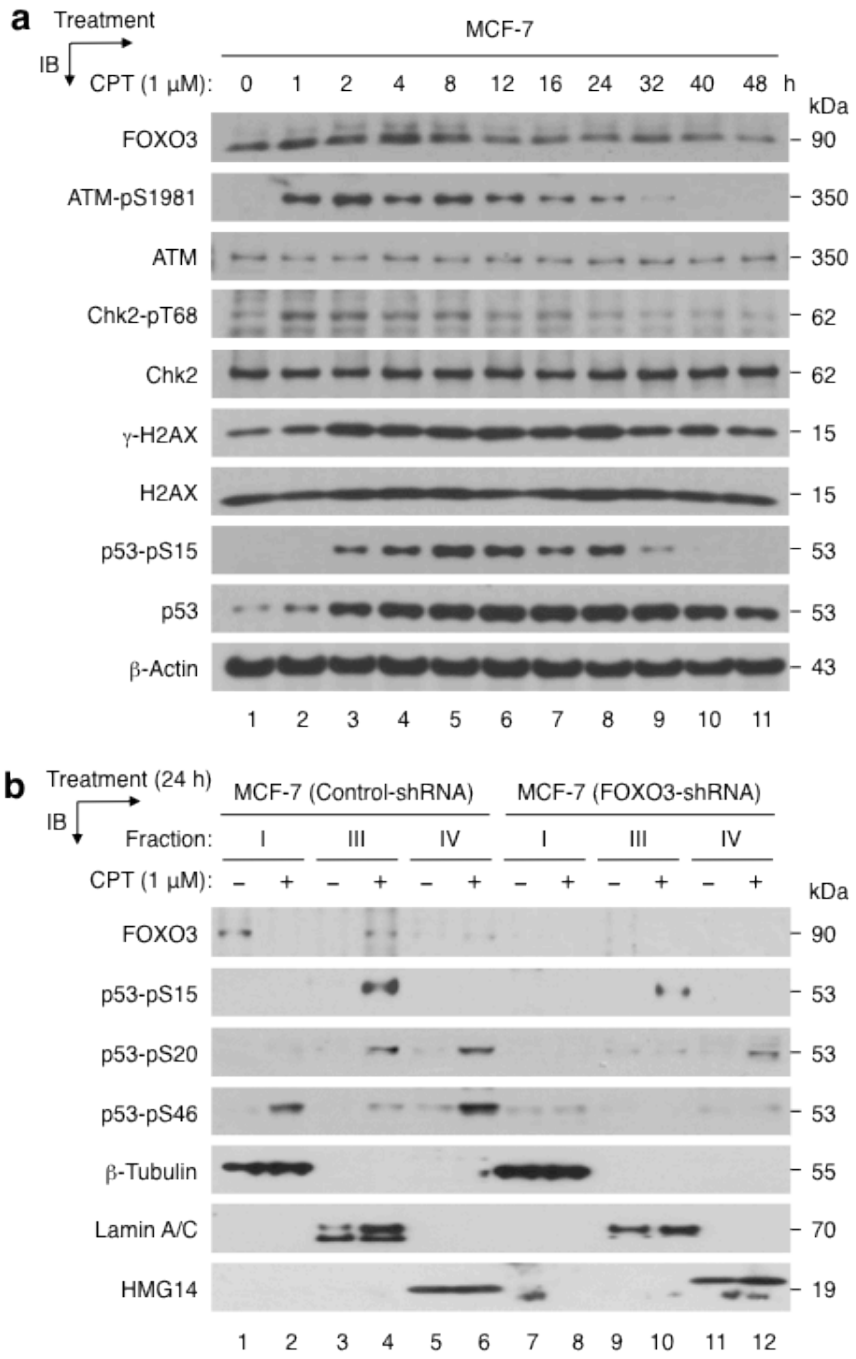
Supplementary Figure S8. Silencing of FOXO3 in A549 and H1299 cells significantly enhance survival after CPT treatment. (**a**, **b**) A549 (a) and H1299 (b) stable cell lines transfected with FOXO3-shRNA or control-shRNA were treated with DMSO control (0) or CPT (1.0 or 0.25 μM) for a time course as indicated (h), and performed cell survival assays by cell counting. The significant P values between the FOXO3-shRNA group versus the control group treated with CPT are indicated. The samples include three biological replicates, the error bars represent standard deviation, and the statistical test is the paired t-test.



Supplementary Figure S9. Overexpression of wild-type p53 promotes CPT-induced apoptosis, whereas overexpression of p53-pS15A or p53-pS20A or p53-pS46A mutant fails to induce apoptosis in the p53-knockdown cells after CPT treatment. MCF-7 (p53-siRNA) cells were transfected with pcDNA3 (control vector) or an expression vector of p53-WT or p53-pS15 or p53-pS20 or p53-pS46 for 48 hours (h). These cells were plated on glass coverslips and treated with low-dose CPT (1 μM) or DMSO control for 60 hours. Then, the cells were fixed on coverslips for determining cellular apoptosis by TUNEL assays using the DeadEnd™ Fluorometric TUNEL System (Promega). Nuclei were stained with DAPI (color-inverted to red). Merged images (yellow) were considered as apoptotic cells and counted under a confocal laser scanning microscope (Leica SP2 AOBS). An average (%) of TUNEL-positive (apoptotic) cells was determined and shown in Fig. 5e. Scale bar: 20 μm.



Supplementary Figure S10. (**a**) H1299 (p53-null) cells were transfected with pcDNA3 (control vector) or an expression vector of p53-WT or p53-pS15 or p53-pS20 or p53-pS46 for 48 h, and the transfected cells were treated with CPT (1 μM) or DMSO control for 60 h. Then, cellular apoptosis was determined by TUNEL assays, and the apoptotic cells were counted as described above. Nuclei were stained with DAPI. Scale bar: 20 μm. (**b**) An average (%) of TUNEL-positive (apoptotic) cells was determined and shown in the diagram. **, P= 0.0001 between the p53-WT group versus the pcDNA3 or p53-S15A or p53-S20A or p53-46A group treated with CPT. The number of biological replicates is three, the error bars represent standard deviation, and the statistical test is the paired t-test. (**c**) H1299 cells were transfected with pcDNA3 (control) or p53 wild-type (WT) or p53 mutants (p53-S15A or p53-S20A or p53-46A) for 48 h, and then treated with CPT (1 μM) (+) or DMSO (-) for 48 h. Whole cell lysates were prepared from these treated cells and the levels of PARP-1 and its degraded proteins, ATM, Chk2, p53, and their phosphorylated proteins were analyzed by immunoblotting with the indicated Abs or an anti-β-actin.



Supplementary Figure S11. (**a**) The amount of FOXO3 protein remains at a similar level between 0 and 16 hours (h) or 48 h after CPT treatment. MCF-7 cells were treated with CPT for full time-course of CPT treatment, 0–48 h, or control DMSO (0 h). The levels of FOXO3, ATM-pS1981, Chk2-pT68, γ-H2AX, p53-pS15 and their total proteins in whole lysates were analyzed by immunoblotting (IB) with the indicated Abs. (**b**) The localization of FOXO3 to the chromatin is significantly decreased at late time treatment with CPT. MCF-7 (control-shRNA) and MCF-7 (FOXO3-shRNA) stable cell lines were treated with CPT for 24 h, then cells were subjected to chromatin fractionation, as described in the Experimental Procedures. Equal amount (20 μg) of each fraction was analyzed by IB with the highlighted Abs. Proteins β-tubulin, lamin A/C, and HMG14 (a chromosome binding protein) represent the fractionation and loading controls of the cytosol (fraction I), the nucleoplasm (fraction III) and the chromatin (fraction IV), respectively.

Supplementary Table S1

shRNA sequences (5'→3') against human FOXO3

HuSH 29mer FOXO3 shRNA (Origene)	CCAGAGCCGTCCGCGATCCTGTACGTGGC
	GTTCGCTGGCCGCACGTCTTCAGGTCCTC
	ATGGCAAGCACAGAGTTGGATGAAGTCCA
	ACAGCACGGTGTTCGACCTTCATCTCTG

Supplementary Methods

Plasmid constructs and DNA transfection

Human p53 wild-type construct (CMV-p53-WT) and its serine mutant constructs CMV-p53-S15A and CMV-p53-S20A were kindly provided by T. Unger (the Weizmann Institute of Science, Rehovot, Israel)[15] and D.W. Meek (Medical Research Institute & Dundee Cancer Centre, Dundee, UK), and CMV-p53-S46A has been described previously[16]. MCF-7 or H1299 cells were transfected with the CMV-p53-WT, or the inactive mutant CMV-p53-S15A, CMV-p53-S20A, and CMV-p53-S46A constructs or control pcDNA3 vector by liposome using GenJet™ In Vitro DNA Tranfection Reagent. For chromatin retention assay, MCF-7 (control-shRNA) and MCF-7 (FOXO3-shRNA) cells were transfected with 300nM control or p53 siRNA (Santa Cruz Biotechnology, sc-44231 or sc-29435) using GenJet reagent. The transfected cells were cultured for 48 hours and transfected with the control pcDNA3 vector or CMV-p53-WT or the p53 constitutively active mutant vectors expressing p53-S15D, p53-S20D, and p53-S46D for 36 hours. Then, all transfected cells were treated with CPT (1 μM) or DMSO (control) for 4 hours and then subjected to chromatin retention assays.

Cytoplasmic and nuclear fractionation

Cells were treated with CPT (1 μM) or control (DMSO) for 2 and 4 hours. Cells were lysed in a lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl2, 0.5% NP-40, 1 mM Na3VO4, 1 μM phenylmethanesulphonyl-fluoride, and 0.15 units/ml aprotinin) and homogenized by 30 strokes in a tightly fitting Dounce homogenizer. The homogenate was centrifuged at 1,500 x *g* for 5min to sediment the nuclei. The supernatant was then resedimented at 15,000 x *g* for 5min, and then the resulting

supernatant formed the cytoplasmic fraction. The nuclei was washed three times and resuspended in PBS containing 0.5% NP-40 to extract nuclear proteins. The extracted material was sedimented at 15,000 x *g* for 10 min and the resulting supernatant was termed the nuclear fraction. Protein concentrations in the cytoplasmic and nuclear fractions were determined with the Bradford protein determination reagent (Bio-Rad Laboratories, Hercules, CA) or BCA Protein Assay Reagent A (PIERCE, Rockford, IL) using BSA as a standard.

MTT assays and cell counting

Cells (1 x 10^3/well) were seeded into 96-well plates and incubated at 37°C in a CO_2 incubator overnight. Cells were treated with DMSO (control) and various doses of CPT as indicated for 24 hours, followed by the addition of 20 μl of MTT solution (5 mg/ml in phosphate buffer). After incubation for 2 hours, a blue crystalline precipitate was dissolved in DMSO (200 μl /well). The visible absorbance at 560 nm of each well was quantified using a microplate reader. For cell counting, cells (1 x 10^5/well) were seeded into 6-well plates and treated with DMSO or CPT as described above for a time course (0 - 4 days). Cell numbers were measured by using a hemocytometer. The relative (%) cell survival rate was calculated by dividing cell number of CPT treatment by that of DMSO treatment.